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Heterozygous β-Thalassemia With Thalassemia Intermedia Phenotype

D. Gasperini,[1] L. Perseu,[2] M.A. Melis,[1] L. Maccioni,[1] M.C. Sollaino,[1] E. Paglietti,[1] A. Cao,[1] and R. Galanello[1]*

[1]Istituto di Clinica e Biologia dell'Età Evolutiva, Università degli Studi di Cagliari, Italy
[2]Istituto di Ricerca sulle Talassemie e Anemie Mediterranee, CNR, Cagliari, Italy

In this study we investigated the molecular bases of the β-thalassemia intermedia phenotype in six patients belonging to two unrelated families of Sardinian descent. Sequence analysis of the β globin gene from these patients detected, as the sole abnormality, the heterozygosity for the codon 39 nonsense mutation. The Aγ and Gγ promoters as well as the HS2 and HS3 core sequences of the β globin LCR from these patients, did not show any non-polymorphic nucleotide variation from the consensus sequence. One of the parents was heterozygous for codon 39 nonsense mutation but showed the β-thalassemia carrier phenotype; the other was hematologically normal and had an entirely normal β globin gene sequence. In both families, other members showed the typical hematological phenotype, clinically silent, of heterozygous β thalassemia. To explain the thalassemia intermedia phenotype, we postulated the presence of an unknown molecular defect interacting with the β globin gene mutation. Haplotype analysis excluded that this postulated defect lies in the β globin gene cluster. Am. J. Hematol. 57:43–47, 1998.

Key words: thalassemia carrier; thalassemia intermedia; β globin gene

INTRODUCTION

Thalassemia intermedia is the clinical definition in use for a spectrum of clinical conditions ranging in severity from the asymptomatic carrier state to the transfusion-dependent thalassemia major. This mild phenotype may result from homozygosity for mild β thalassemia mutations, compound heterozygosity for mild and severe β thalassemia mutations, coinheritance with homozygous β thalassemia of α thalassemia or hereditary persistence of fetal hemoglobin (HPFH), double heterozygosity for β thalassemia, and triple α globin gene arrangement or the presence of a highly unstable hemoglobin variant [1,2].

Herein, we report six individuals belonging to two unrelated families of Sardinian descent, with the clinical phenotype of thalassemia intermedia, in whom sequence analysis of the β globin gene detected, as the sole abnormality, heterozygosity for codon 39 nonsense mutation, the other β globin gene being entirely normal. To explain the thalassemia intermedia phenotype, we postulated the presence of an unknown molecular defect interacting with the β globin gene mutation. Haplotype analysis excluded that this postulated defect lies in the β globin gene cluster.

MATERIALS AND METHODS

Subjects

Six members with thalassemia intermedia and their relatives from two unrelated Sardinian families have

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*Correspondence to: Renzo Galanello, Istituto di Clinica e Biologia dell'Età Evolutiva, Università degli Studi di Cagliari, Via Jenner s/n, Cagliari, Italy. E-Mail: RGALANEL@MCWEB.UNICA.IT

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been studied. Peripheral blood samples were obtained as part of the clinical study after informed consent.

Hematological Analysis

Red blood cell indices were determined with Coulter Max-M (Coulter Electronics, Hialeah, FL). Hemoglobin A_2 and F quantitation was performed by high-pressure liquid chromatography (HPLC-VARIANT, Bio-Rad, Milan, Italy) [3]. Globin chain synthesis analysis was carried out on peripheral blood reticulocytes [4]. Ferritin was determined with an ELISA method (RAMCO Lab, Houston, TX).

DNA Analysis

DNA was extracted from peripheral blood leukocytes and analysed by several methods. The α globin gene arrangement was determined by Southern blot analysis with the restriction enzymes Bam HI or Bgl II and hybridization with α and ζ globin gene probes, respectively [4].

The β globin gene from position −670 5′ to the CAP site to position +189 3′ to the termination codon was amplified in a 2,334-kb fragment by polymerase chain reaction (PCR) [5] using the following primers: forward 5′TGCACAGAGCACATTGAT3′ and reverse 5′CACTGACCTCCCACATTCCC3′. To amplify the hypersensitive sites HS2 and HS3 of the Locus Control Region (LCR) [6,7] the primers used were: forward 5′TAGTCCAAGCATGAGGAGTTC3′ and reverse 5′TTCTATGTATAGAGGCCACCT3′ (HS2); forward 5′AGCCATGAAGAAGTCTATGAC3′ and reverse 5′GGGAGCAGTCCCATGTAGTAG3′ (HS3).

The 470 base pairs (bp) from HS2 and 369 bp from HS3 amplification products include GATA-1, NF-E2, and CACC binding boxes [8,9].

DNA sequencing analysis of β globin gene, HS2 and HS3 of LCR was performed by the dideoxy-chain termination method of Sanger et al. [10] on amplified single-strand DNA using the enzyme T7 DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) [11].

β globin cluster haplotypes, according to Orkin et al. [12], have been defined by PCR-based methods [13,14]. Seven fragments each containing one of the following polymorphic sites: Hinc II (ε), Hind III (Gγ and Aγ), Hinc II (5′ and 3′ ψβ), Ava II (5′β), and Hinf I (3′β), were amplified using specific primers and subsequently digested with the appropriate restriction enzyme. The fragments obtained after digestion, were separated by electrophoresis on agarose gel containing ethidium bromide and visualized under ultraviolet light. The promoter of Gγ and Aγ gene was analysed by denaturing gradient gel electrophoresis (DGGE) as previously described [15].

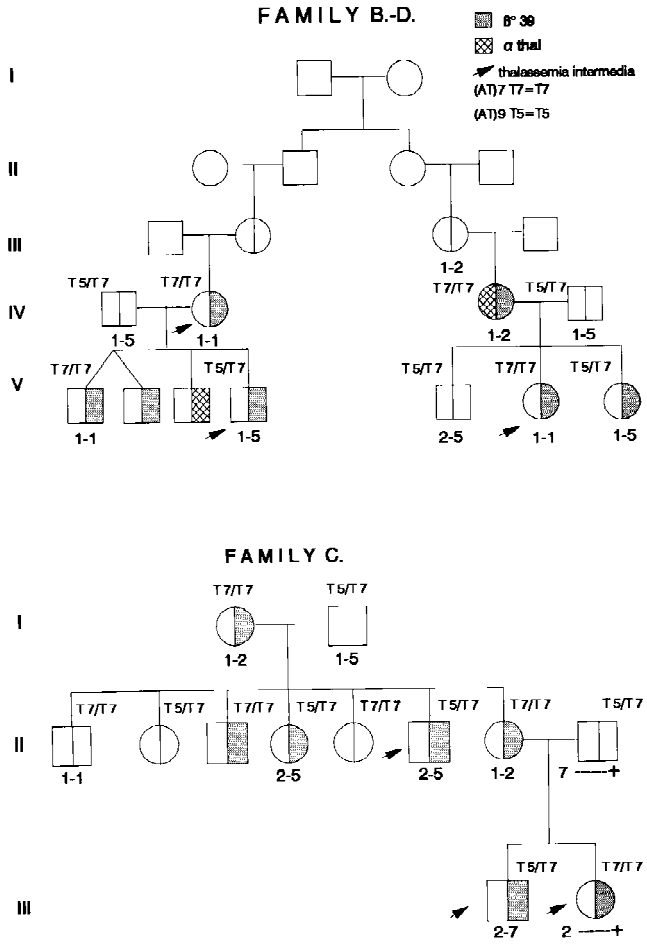

Fig. 1. Pedigrees of the families. Haplotype numbers according to Orkin et al. [12] are reported below the subjects.

RESULTS

Families Studies

The pedigrees of the families C and B-D are shown in Figure 1 and the haematological features in Table I. In patients with thalassemia intermedia the reported red blood cell indices, bilirubin, erythroblasts, and reticulocytes are the mean values of several determinations (4 to 6) in the last 2 years. We considered patients as thalassemia intermedia when they present moderate to severe anemia (Hb < 10 g/dl), splenomegaly, hyperbilirubinemia, erythroblasts in peripheral blood smear, and an α/non α ratio higher than 2.5 (absolute criteria), possibly associated with mild to moderately elevated HbF level, reticulocytosis, thalassemia-like bone modifications, and liver enlargement. Three individuals in family C (II-6, III-1, and III-2) and three (IV-2, V-4, V-6) in family B-D, fulfill the above criteria. They have, in fact, moderate to severe microcytic anemia with Hb levels varying from 7.2 to 9.0 g/dl, hepatosplenomegaly, mild thalassemia-like facial bone modifications, hyperbilirubinemia, marked red blood cell morphological abnormalities (any-

TABLE I. Hematological Features of the Families*

	Age (year)	Hb (g/dl)	MCV (fl)	MCH (pg)	HbA_2 (%)	HbF (%)	α/β+γ (ratio)	Ferritin (ng/ml)	NRBC	Total bilirubin (μmol/l)	Reticulocytes (%)
Family B-D											
III-2		13.1	80.8	27.9	2.5	0.8					
III-3		13.7	87	30.0	2.3	0.7					
IV-1		14.7	81.4	27.6	2.4	0.8	0.9				
IV-2	**43**	**7.2**	**58.2**	**18.0**	**4.2**	**2.4**	**4.0**	**470**	**7**	**36**	**4.9**
IV-3		9.5	61.4	20.1	4.9	2.0	2.5				
IV-4		15.4	92	34.0	2.6	0.9	0.9				
V-1		11.6	61	20.4	4.8	0.6					
V-2		10.3	59	19.4	4.7	0.7					
V-3		11.5	65	22.9	2.3	0.1					
V-4	**4**	**8.6**	**57**	**17.8**	**5.0**	**7.2**		**77**	**2**	**24**	**2.7**
V-5		13.5	85	27.2	3.1	0.2					
V-6	**13**	**7.8**	**61**	**19.8**	**4.6**	**4.0**	**4.0**	**127**	**4**	**53**	**3.4**
V-7		10.7	59	19.5	5.5	5.0					
Family C											
I-1		11.3	56	19.0	4.8	8.5	2.1				
I-2		14.7	84.0	26.8	2.3	0.8	1.1				
II-1		14.7	82.0	29.0	2.5	0.8					
II-2		14.4	91	29.7	2.8	1.3					
II-3		14.1	75	22.6	5.7	1.4					
II-4		12	69	21.5	5.5	3.2					
II-5		12	71	21.3	2.2	1.6					
II-6	**18**	**9**	**65**	**18.4**	**4.7**	**19.4**	**3.14**		**10**	**58**	**6.2**
II-7		11	57	19.0	4.7	6.4	1.9				
II-8		13.8	87	32.6	2.4	0.5	1.1				
III-1	**13**	**8.2**	**58**	**19.9**	**5.7**	**5.9**	**3.9**	**165**	**7**	**43**	**4.5**
III-2	**6**	**9.0**	**60**	**19.7**	**5.6**	**10.2**		**93**	**5**	**24**	**4.6**

*NRBC = nucleated red blood cells/100 leukocytes. Thalassemia intermedia patients are in bold.

socytosis, poikilocytosis, basophilic stippling), reticulocytosis, and erythroblasts in the peripheral blood film. MCV and HbA_2 were in the range of the β thalassemia carriers (MCV = 57–65 fl, HbA_2 = 4.2–5.7%). HbF levels were increased in most of the patients (range = 2.4–19.4%). The α/non α ratio was markedly unbalanced (3.1 in II-6, 3.9 in III-1 from family C, and 4.0 in IV-2 and 4.0 in V-6 from family B-D). Patient V-6 from family B-D had been splenectomized at age of 12 because of worsening of anemia. Patient IV-2 of the same family was transfused sporadically during pregnancies because of severe reduction of hemoglobin levels. Ferritin was increased in all patients. Although subject IV-3 from this family showed a moderate anemia (9.5 g/dl), she could not be considered as thalassemia intermedia since the spleen was not enlarged, erythroblasts in the peripheral blood were absent, ferritin was normal (51 ng/ml), and reticulocytes were only mildly elevated (2.1%). However, it should be pointed out that she also coinherited α-thalassemia (-α/αα genotype), which results in a lower globin chain imbalance (α/β ratio = 2.5) and could be the reason for the milder phenotype.

In family C, the mothers (I-1 and II-7) of the patients and two other members of the family (II-3, II-4), showed the classical phenotype of heterozygous β thalassemia, except for the presence of a moderate increase in HbF levels (from 1.4 to 8.5%). The fathers (I-2 and II-8) of the three patients from family C were hematologically normal with a normal α/β biosynthetic ratio (Table I). In family B-D the fathers (IV-1) and (IV-4) of the patients V-4 and V-6 were hematologically normal and both also had a balanced α/β globin synthesis ratio (Table I). The fathers of IV-2 and IV-3 were not available, but from the pedigree's study they were most likely β-thalassemia carriers. Also in this family, other members (V-1, V-2, V-7) showed only the typical hematological phenotype, clinically silent, of heterozygous β thalassemia.

The association with a red cell membrane defect was excluded by appropriate studies.

DNA Analysis

β globin gene sequence analysis was carried out in all members of the families with thalassemia intermedia or β-thalassemia carrier phenotype, as well as in the hematologically normal parents of four of the patients (I-2 and II-8 from family C; IV-1 and IV-4 from family B-D). β globin gene analysis from all the patients showed the presence of the codon 39 nonsense mutation in one of the β globin genes, and completely normal sequence in the other. In heterozygotes for β-thalassemia from these

families, we detected the presence of heterozygosity for codon 39 nonsense mutation; the other β globin gene was again entirely normal. Normal β globin gene sequence was also detected in the available fathers (I-2 and II-8 from family C; IV-1 and IV-4 from family B-D) of patients with thalassemia intermedia.

In order to investigate further the molecular basis for thalassemia intermedia in these patients, we have analyzed the configuration of the polymorphic (AT)n (T)n sequence at position −530 5′ to the β globin gene, because the (AT)9 (T)5 motif at this position has been found in previous studies associated with silent β-thalassemia [16,17]. In family C (Fig. 1) we detected the (AT)9 (T)5 motif in a heterozygous state in both subjects with thalassemia intermedia, in one of the typical β-thalassemia carriers (II-4), and in both the normal fathers of the patients. In all the other members including two β-thalassemia carriers (I-1 and II-3) we detected the common (AT)7(T)7 configuration. In family B-D (Fig. 1), the patients IV-2 and V-6 showed the homozygous (AT)7 (T)7 configuration, while V-4, the other patient with thalassemia intermedia phenotype, had the configuration $(AT)_9$ T5/ $(AT)_7$ T_7.

α globin gene mapping excluded the presence of the triple α globin gene arrangement in both families. In family C, DGGE analysis of the Aγ and Gγ promoter revealed normal sequences. We also sequenced the HS2 and HS3 core sequences of the Locus Control Region in the patients with thalassemia intermedia phenotype. The HS3 sequence was entirely normal in patients from both families. The AT-rich region of HS2 (9) showed the (AT)10 configuration in patients from family B-D, and the (AT)8 in those of family C (data not shown). Haplotype analysis (Fig. 1) showed that the $\beta^{\circ 39}$ mutation was associated with haplotype 2 in family C and haplotype 1 in family B-D (haplotype number according to Orkin et al. 1982; [12]). In family B-D the normal β globin gene of thalassemia intermedia patients was contained in haplotype 1 in IV-2 and V-6, and in haplotype 5 in V-4. In family C, the normal β globin gene was associated with haplotype 5 in II-6, with haplotype 7 in III-1, and with - - - - - - + haplotype in III-2.

DISCUSSION

This study describes two unrelated families of Sardinian descent in which six members showed the phenotype of thalassemia intermedia. β globin gene analysis revealed heterozygosity for codon 39 nonsense mutation whereas the β globin gene sequence of the in trans β globin gene was entirely normal. To explain the unusually severe phenotype for a β thalassemia carrier, we postulated the existence in these patients of an unknown genetic determinant which, interacting with the β thalassemia trait, produced the phenotype of thalassemia intermedia. We easily excluded that the postulated determinant resides within the β globin gene cluster in cis to the $\beta^{\circ 39}$ mutation, because other members of the family, heterozygotes for the $\beta^{\circ 39}$ mutation, had the typical phenotype of the β thalassemia carrier state. This genetic determinant may lie in the β globin gene cluster in trans to the $\beta^{\circ 39}$ mutation or elsewhere in the genome. A family in which the β-thalassemia phenotype segregates independently of the β globin gene cluster has been recently reported by Thein et al [18]. The existence of a silent β thalassemia mutation was excluded because the apparently normal parents of subjects with thalassemia intermedia had a normal α/β ratio and showed normal β globin gene sequences. Sequence analysis of the Aγ and Gγ promoters and of the HS2 and HS3 core sequences of LCR failed to detect any sequence variation from normal.

We have also analyzed the configuration of the polymorphic (AT)n (T)n sequence at position −530 5′ to the β globin gene and found in both patients from family C the (AT)9 (T)5 motif, which, in previous studies, has been detected in association with the β thalassemia silent carrier phenotype [16, 17]. However, the (AT)9 (T)5 motif was also detected in member II-4 of the same family, who was heterozygous for the $\beta^{\circ 39}$ mutation, as the patients with thalassemia intermedia, but showed a typical clinically silent β thalassemia carrier phenotype. These findings excluded any role of the (AT)9 (T)5 motif in producing the β thalassemia intermedia phenotype in family C. In support of this conclusion, also in family B-D two thalassemia intermedia patients (IV-2 and V-6) had only the (AT)7 (T)7 motif. Furthermore, we have recently found the (AT)9 (T)5 motif in individuals with entirely normal hematological features, hemoglobin pattern, and balanced globin chain synthesis [19].

In each family, the patients with thalassemia intermedia inherited different chromosome haplotypes containing the normal β globin gene. This indicates that the postulated genetic determinant responsible for the severe phenotype does not reside within the β globin gene cluster in trans. Furthermore, in both families the α globin gene arrangement was entirely normal. We may speculate that the thalassemia intermedia phenotype in our patients may result from double heterozygosity for a β thalassemia mutation and another defect located outside the β globin gene cluster, which may involve the function of a gene encoding for a transcription factor regulating the function of the β globin gene. A prime candidate for the postulated mutation is the gene encoding erythroid Kruppel-like factor (EKLF), an erythroid specific DNA protein that activates transcription from the β globin CACCC element [20]. In this context, it should be noted that thalassemia mutations residing in this element prevent EKLF binding and promoter activation [21]. Furthermore, EKLF knock-out mice exhibit selective β glo-

bin deficiency and die prenatally from anemia, simulating closely the phenotype of thalassemia major [22].

The concept of a β thalassemia phenotype resulting from defect(s) outside the β globin gene cluster is not new. In a group of patients with α thalassemia associated with mental retardation (ATR), indeed, the disorder maps to the X chromosome and is referred to as the ATR-X syndrome [23]. Positional cloning led to the identification of a gene coding for a helicase, which was found to be mutated in the ATR-X syndrome [24, 25].

We have previously described a family of Central Italian ancestry in which three members heterozygous for the $\beta^{\circ 39}$ mutation in two generations displayed the phenotype of thalassemia intermedia [17]. We postulated also in this family the presence of another molecular defect lying outside the β globin gene. This defect was present in the father of two of the patients who showed thalassemia-like red cell indices, normal HbF, low HbA_2, and unbalanced α/non α chain synthesis. Further studies are in progress to define the molecular bases for β thalassemia intermedia in these interesting patients.

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